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Temporal trends of halogenated and organophosphate contaminants in striped dolphins from the Mediterranean Sea

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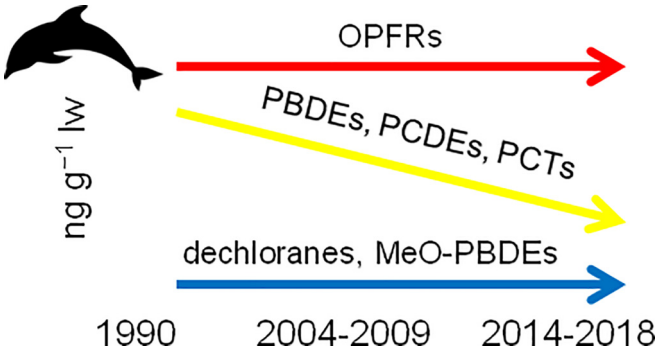
HIGHLIGHTS

- Target and non-target analysis to monitor halogenated/organophosphate pollutants
- Muscles from 42 striped dolphins from three different periods were analysed.
- PCDEs and PCTs were determined for the first time in dolphin samples.
- PBDE, PCDE and PCT declined from 1990 to 2018, and OPFRs had a steady concentration.
- Despite level decrease, these compounds remain a risk to Mediterranean dolphins.

GRAPHICAL ABSTRACT



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ABSTRACT

PBDEs, HBCD, novel DBDPE, PBEB and HBB, dechloranes, OPFRs and natural MeO-PBDEs were monitored in muscle of striped dolphins (*Stenella coeruleoalba*) from the Mediterranean Sea collected in three time periods (1990, 2004–2009 and 2014–2018). PBDEs levels decreased about 60% in under three decades, from 5067 ± 2210 to 2068 ± 2642 ng g^{-1} lw, evidencing the success of their ban. Most PBDEs were found in all the samples, with BDE-47, -99, -154, -100 and -153 as the main contributors. Found in 71.4% of the samples, α-HBCD was stable through time and usually <LOQ. DBDPE concentrations decreased by 89% from 1990 to 2004–2009 and have remained stable since. HBB occurred rarely and decreased by 94% to a current few ng g^{-1} lw. Dec 602 was the main dechlorane with stable concentrations around 1200 ng g^{-1} lw, but a declining trend might have started in the last years. OPFRs concentrations were stable and showed the highest concentrations of all FRs in 2014–2018: 6253 ± 11,293 ng g^{-1} lw. TBOEP and TNBP contributed to most of the OPFR concentration, the former with decreasing levels by 96%. MeO-PBDEs showed mean concentrations between 600 and 700 ng g^{-1} lw in all periods. Non-targeted analysis allowed the identification and semi-quantification of additional chlorinated pollutants, such as polychlorinated terphenyls (PCTs) (levels decreasing by 81% to a current 770 ng g^{-1} lw mean) and polychlorinated diphenyl ethers (PCDEs) (decreasing by 83% to a current 3200 ng g^{-1} lw) in Mediterranean marine mammals for the first time.

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1. Introduction

Nowadays there is an obvious concern about the human impact on the environment, including marine pollution. Regulation such as the Stockholm Convention on Persistent Organic Pollutants (POPs) and European directives have restricted or banned the use of some contaminants of concern. The impact of these regulations should translate into decreasing time trends for the regulated contaminants and alternative compounds should appear. Cetaceans are long-lived, highly mobile top predators that have been used as bioindicators of the environmental pollutant loads and temporal trends because their tissues integrate the environmental characteristics of the water masses in which they live (Borrell and Aguilar, 2007).

During the last decade, polybrominated diphenyl ethers (PBDEs) have been added to the Stockholm Convention and their use has been restricted or banned by several European directives and regulations (REACH, 2006; HSEEE, 2011; PSWP, 2013). PBDEs are flame retardants (FRs), which are applied to all kind of materials to prevent them from ignition. In the past, PBDEs have been the most used FRs (Alaee et al., 2003). They are available as mixtures at different levels of bromination. PBDEs leach out of materials since they are not bonded but simply blended with the polymers (Alaee et al., 2003). Hence, PBDEs have been found in environmental (Guerra et al., 2010; Sánchez-Avila et al., 2011) and biological matrices (Lacorte et al., 2010). PBDEs persist in the environment, bioaccumulate through the food web and show toxicity (Mikula and Svobodova, 2006).

Hexabromocyclododecane (HBCD) is another FR than can leach out of the materials and is toxic to aquatic organisms, albeit less than PBDEs (Alaee et al., 2003; SC, 2008; Lu et al., 2018). It has been detected in environmental and biotic samples (Eljarrat et al., 2004a; Eljarrat et al., 2009) and included in the REACH regulation and the Stockholm Convention.

Substitutes for the banned FRs include decabromodiphenyl ethane (DBDPE), pentabromoethylbenzene (PBEB) and hexabromobenzene (HBB) (Hardy et al., 2002; Covaci et al., 2011; Cruz et al., 2015). Additionally, Dechlorane Plus (DP) and dechloranes 602, 603 and 604 (Dec 602, Dec 603, Dec 604) are chlorinated alternatives to Mirex. These alternative compounds have been also found in environmental matrices and biota (Hong et al., 2010; Guerra et al., 2011; Aznar-Alemany et al., 2017a, 2017b; Giulivo et al., 2017).

A growing alternative to halogenated flame retardants (HFRs) are organophosphorus flame retardants (OPFRs). They represented 20% of all FRs used in Europe in 2006; twice the quantity of brominated FRs (Van der Veen and de Boer, 2012). OPFRs are also released from materials and access environmental matrices through washout, infiltration, deposition, etc. (Schreder and La Guardia, 2014). Moreover, OPFRs are used as plasticisers, so they leak from the tons of plastic that reach the seas and oceans. Their presence has been reported in sediments, water, fish and marine mammals (Giulivo et al., 2016; Sala et al., 2019; Garcia-Garin et al., 2020) and their toxicity has been reported (Van der Veen and de Boer, 2012).

Methoxylated PBDEs (MeO-PBDEs) are natural analogues to PBDEs that are synthesized by some marine organisms (Malmvarn et al., 2008). They have been found in cetaceans and seafood (Alonso et al., 2014; Aznar-Alemany et al., 2017a, 2017b). Being natural compounds, their presence cannot be controlled. However, their structural similitude to PBDEs might confer them similar negative effects that would add to those of the anthropogenic FRs. As they can be analysed using the same method, it makes sense to collect the data.

This study assesses the occurrence of the aforementioned FRs in striped dolphins (*Stenella coeruleoalba*) from the Mediterranean Sea through three time periods. A non-targeted analysis for halogenated contaminants was also performed in search for compounds that occurred frequently and at apparent great concentrations and that might be currently overlooked. Some suspects were confirmed and semi-quantified.

2. Materials and methods

2.1. Sampling

Samples of dorsal muscle were collected from 42 striped dolphins that were found stranded on the Catalan coast, in the North Western Mediterranean Sea. The samples were collected in three different periods, including 15 samples from 1990, 15 samples from 2004 to 2009 and 12 samples from 2014 to 2018. All individuals were adult males ranging from 164 to 224 cm of body length (Calzada et al., 1997). Samples were kept frozen at −20 °C and were freeze-dried prior to analysis. Lipid content referenced to wet weight (ww) was mostly between 0.25 and 1.21%, with three samples between 3.98 and 6.51% (see Table 1 for details). The nature of these samples makes it impossible to design a sample plan choosing all the characteristics of the samples. The co-authors from the Universitat de Barcelona collect the dolphin samples as part of a monitoring programme. Having adult male and three different time periods as key requirements, samples were selected when there were at least 5 g available.

2.2. Standards and reagents

Native and ^{13}C-labelled standard mixtures of PBDEs (both including BDE-28, -47, -99, -100, -153, -154, -183 and -209); *syn*-DP, *anti*-DP and ^{13}C-*syn*-DP; α-, β- and γ-HBCD and their deuterated congeners were purchased from Cambridge Isotope Laboratories Inc. (Andover, MA, USA). Dec 602 (95%), Dec 603 (98%) and Dec 604 (98%) were purchased from Toronto Research Chemical Inc. (Toronto, ON, Canada). HBB, DBDPE, PBEB and the standard mixture of MeO-PBDEs (5-MeO-BDE-47, 6-MeO-BDE-47, 4′-MeO-BDE-49, 2′-MeO-BDE-68, 5′-MeO-BDE-99, 5′-MeO-BDE-100, 4′-MeO-BDE-101 and 4′-MeO-BDE-103) were bought from Wellington Laboratories Inc. (Guelph, ON, Canada). Tris (2-butoxyethyl) phosphate (TBOEP), tris(chloroethyl) phosphate (TCEP), tris(chloroisopropyl) phosphate (TCIPP), trihexyl phosphate (THP) and tris(2-ethylhexyl) phosphate (TEHP) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Isodecyldiphenyl phosphate (IDPP) and 2-ethylhexyldiphenyl phosphate (EHDPP) were purchased from AccuStandard (New Haven, CT, USA). Diphenylcresyl phosphate (DCP), tri-*n*-butyl phosphate (TNBP), triphenyl phosphate (TPHP), triphenylphosphine oxide (TPPO) and tris(1,3-dichloro-2-propyl) phosphate (TDCPP) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Tricresyl phosphate (TMCP) was purchased from Dr. Ehrenstorfer (Augsburg, Germany). Isopropyl phenyl phosphate (IPPP) was purchased from Chiron (Trondheim, Norway). Labelled d_{15}-TDCPP, d_{27}-TNBP, d_{12}-TCEP and $^{13}C_2$-TBOEP were purchased from Wellington Lab-oratories Inc. (Guelph, ON, Canada). Labelled d_{15}-TPHP was obtained from Cambridge Isotope Laboratories Inc. (Andover, MA, USA). ^{13}C-Labelled standards for the non-targeted analysis (PCB-101, PCB-118, PCB-153, BDE-47, BDE-99 and BTBPE) were purchased from Wellington Laboratories Inc. (Guelph, ON, Canada). To confirm the suspected compounds, 3,3′,4,4′-tetrachlorodiphenyl ether (CDE-77), 2,2′,4,4′,5-pentachlorodiphenyl ether (CDE-99), 2,2′,4,4′5,5′-hexachlorodiphenyl ether (CDE-153) and polychlorinated terphenyl mixtures Aroclor 5432 and Aroclor 5460 were obtained from AccuStandard. Dichloromethane (DCM), methanol and sulphuric acid were purchased from Merck (Darmstadt, Germany). Acetone and hexane for organic trace analysis were purchased from J.T. Baker (Center Valley, PA, USA).

Table 1
Sampling information.

Year	*n*	Size (cm)	Water content (%)	Lipids (referred to ww) (%)
1990	15	181–216	65.1–73.8	0.25–1.18
2004–2009	15	166–224	66.3–72.1	0.26–1.21, *3.98, 6.51*
2014–2018	12	164–210	71.0–73.6	0.19–1.07, *5.88*

2.3. Sample preparation

For HFRs and MeO-PBDEs, sample extraction was carried out according to previous works (de la Cal et al., 2003; Labandeira et al., 2007). Freeze-dried sample (1.5 g dry weight (dw)) was spiked with the labelled standards. Pressurized liquid extraction (PLE) was used with hexane:dichloromethane (1:1). Lipid content was determined gravimetrically weighting the dried extract every 30 min until steady weight. The extract underwent an acid attack to remove the fat. The organic phase was cleaned by solid phase extraction (SPE) using Al–N cartridges (5 g) and eluted with hexane:dichloromethane (1:2). The extract was reconstituted with 40 μL of toluene.

The extraction of OPFRs was carried out by ultrasound assisted extraction according to an existing method (Giulivo et al., 2016). Freeze-dried sample (0.5 g dw) was extracted by sonication with hexane:acetone (1:1). The extract was reconstituted in 5 mL of hexane: methanol (1:3) and centrifuged and an aliquot of 200 μL was used for the instrumental analysis. Purification was performed on-line at the beginning of the instrumental analysis. Labelled OPFRs standards were added prior to analysis by turbulent flow chromatography (TFC) coupled to LC-MS-MS.

For the non-target analysis, a softer version of the method for HFRs was used, avoiding the acid attack. Freeze-dried sample (1 g dw) was spiked with the labelled standards. The extraction was carried out with a Buchi SpeedExtractor E-914/E-916 using hexane:dichloromethane (1:1) in two cycles of 20 min at 100 °C. The extracts were re-dissolved in hexane before undergoing a SPE using acidic alumina cartridges and eluting with hexane:dichloromethane (1:2). Extracts were evaporated to incipient dryness and reconstituted with toluene to a final volume of 600 μL prior to the instrumental analysis.

2.4. Instrumental analysis

HFRs and MeO-PBDEs (except for HBCD) were analysed by GC–MS-MS using an Agilent 7890A gas chromatograph coupled to an Agilent 7000B triple quadrupole mass spectrometer and a DB-5 ms column. Brominated compounds were analysed using electronic ionization (EI). The instrumental conditions and the spectrometric determination are described in previous publications (Eljarrat et al., 2007; Barón et al., 2014). Due to their low sensitivity with GC-EI-MS-MS, BDE-209 and DBDPE were analysed by GC–MS with the same chromatographic conditions in an Agilent 5975A mass spectrometer using negative ion chemical ionization (NICI) (Eljarrat et al., 2004b). The analysis of dechloranes was performed by GC-NICI-MS-MS as described in a previous article (Barón et al., 2012).

After analysis of the previous HFRs, extracts were re-dissolved in methanol. HBCD was analysed using an Agilent HP 1100 binary pump LC system coupled to a hybrid triple quadrupole/linear ion trap 4000QTRAP (Guerra et al., 2008).

For OPFRs, online sample purification and analysis was performed with a Thermo Scientific TurboFlow™ system (Giulivo et al., 2016). CycloneTM-P and C18-XL columns were used in combination for purification. Chromatographic separation was achieved with an analytical column Purosphere Star RP-18, starting at a flow rate of 0.25 mL/min and performed under gradient elution conditions using 0.1% formic acid in water (A) and ammonium acetate in methanol (B) as mobile phases. Initially, solutions A and B were used at 50%, then the gradient increased up to 80% of B, and finally up to 100% of B. Spectrometric analysis was performed with a triple quadrupole with a heated-electrospray ionization source.

2.5. Quality assurance

Throughout all sampling and analysis processes, plastic material was avoided due to potential contamination, as some of our analytes such as OPFRs are used as FRs but also as plasticizers. However, OPFR contamination can come from different places that cannot be controlled, like indoor or nitrogen from the evaporator. A realistic goal is to minimize as much as possible the blank signal, i.e., heating all the non-volumetric material at 340 °C and rinsing with an appropriate solvent just before use. In any case, for each batch of samples, a blank was included. Blank levels were subtracted from corresponding samples, only if blank signals do not exceeded 10% of sample signals. If blank values are greater than 10%, then the sample is discarded and re-analysed in another batch of samples.

Quality parameters such as recoveries, limits of detection (LODs) and limits of quantification (LOQ) are summarized in *Supporting information* (Table S1).

2.6. Non-targeted analysis

GC × GC-HRQTOFMS analysis was performed with an Agilent 7890B gas chromatograph (Agilent Technologies, CA, USA) fitted with a Zoex ZX2 GC × GC thermal modulator (Zoex, TX, USA) and interfaced to a Waters Xevo G2-XS quadrupole time-of-flight mass spectrometer (Waters Corporation, Wilmslow, UK). The first-dimension column was an Rtx-5 (60 m × 0.25 mm × 0.25 μm film) followed by a Restek Siltek deactivated guard column (1 m × 0.15 mm) in the modulator loop. The second-dimension column was an Rtx-17 SIL (1 m × 0.15 mm × 0.15 μm film) and was placed in a secondary oven. The secondary column was then connected to a Custom MXT tubing (sulfinert treated, 0.8 m × 0.18 mm), which was inserted into the transfer line. Helium was used as the carrier gas and the flow was 1.5 mL min^{-1}. The injector temperature was 280 °C. The initial oven temperature was held at 40 °C for 1 min, and then ramped at 3 °C min^{-1} to 310 °C and held for 10 min. The secondary oven was at 10 °C offset, relative to the primary oven. The modulator was at a 15 °C offset relative to the primary oven and the modulation period was of 4 s. The transfer line was at 340 °C. The cone gas flow rate was 100 L hr^{-1} and the auxiliary gas flow was 150 L hr^{-1}. The ion source temperature was 150 °C. The acquisition range was 50–1200 amu and the acquisition rate was 30 Hz. The mass spectrometer was operated at a resolving power of > 20,000 (FWHM). Data processing was conducted using GC Image HRMS R2.5 (Zoex). Confirmation of compounds identified by non-targeted analysis was performed in the single dimension GC mode using a 15 m db-5ht (0.25 mm × 0.1 um) column and the GC conditions described by Di Lorenzo et al. (2019).

A composite mass spectrum was generated by combining all mass spectra recorded during the chromatographic separation. Mass measurements, recorded using the IUPAC mass scale were converted to the H/Cl mass scale, which is defined by the substitution of a hydrogen atom by a chlorine atom being equal to 34.000 Da (Taguchi et al., 2010). H/Cl mass is calculated by applying a 34/33.96102 factor to IUPAC mass. The H/Cl mass defect plot enables the efficient and comprehensive characterisation of halogenated compounds (Jobst et al., 2013) and helped guide the extraction of mass chromatograms from the GC × GC contour plot (not shown).

The ions with the exact masses of the mass defect plot were extracted from the corresponding TIC of the specific samples. The mass spectra were interpreted and elemental compositions were assigned using the following criteria: C 0–100; N 0–5, O 0–5, S 0–5, Cl 0–12, Br 0–12. The measured and theoretical masses and isotope ratios were within 5 ppm and ± 15% respectively.

2.7. Statistical analysis

As mentioned in the sampling section, the nature of the samples resulted in a small sample size and high standard deviations. While simple data observation would be appropriate, some statistical analysis was performed as a contrast. Since the results of both methods agreed, it was decided to add the statistical information in the results, albeit with this reminder of the limitations of the data set.

Table 2
Contaminant concentrations (ng g^{-1} lw) and frequencies of detection (FD) by periods.

Period		∑PBDEs	DBDPE	HBB	PBEB	α-HBCD	∑Dechloranes	∑OPFRs	∑MeO-PBDEs	∑PCDEs	∑PCTs
1990	Median[a]	4551	<LOQ	56.3	<LOD	<LOD	1050	2810	615	14,500	3600
	Mean[a]	5067	85.6	24.1	<LOD	16.3	1227	7288	626	19,000	4000
	SD[a]	2210	99.1	41.4	–	63.0	754	10,021	198	10,000	2300
	FD (%)	100	100	26.7	0.00	60.0	100	100	100	100	100
2004–2009	Median	2142	<LOQ	1.25	<LOD	<LOD	1050	1227	406	4200	1100
	Mean	2722	9.10	4.30	0.37	358	1244	3980	682	7500	1400
	SD	2435	20.3	9.74	0.87	1135	940	8737	894	7800	1300
	FD (%)	100	86.7	20.0	20.0	73.3	100	100	100	100	100
2014–2018	Median	1041	<LOQ	<LOQ	<LOD	<LOD	318	2513	362	670	340
	Mean	2068	27.7	1.46	<LOD	82.5	1245	6253	687	3200	770
	SD	2642	74.0	4.28	–	153	2246	11,293	774	4400	980
	FD (%)	100	75.0	16.7	0.00	83.3	100	100	100	91.7	100

[a] Means, median and SDs are calculated including all samples, <LOQ values as equal to the LOD and <LOD values as 10% of the LOD.

The data were divided into three groups according to the sampling period. The results for each pollutant family and the sampling period were checked first to detect possible outliers. Extreme outliers, which differ in at least 3 IQR units beyond Q1 and Q3, were excluded from the statistical analysis. The normality within the groups and the homoscedasticity between the groups were evaluated using the Kolmogorov-Smirnov and Levene tests, respectively. All the compounds showed a normal distribution in each group of samples and only three of them showed homoscedasticity between the groups.

For each family of compounds separately, differences between sampling periods were investigated using one-way analysis of variance (ANOVA), followed by multiple comparisons based on the Tukey HSD post hoc test for compounds with homogeneity of variances (i.e. PBDEs and HBB and dechloranes) and the Games-Howell post hoc test for compounds with variance heterogeneity (i.e. OPFRs, PCDEs, PCTs, MeO-PBDEs). For those families of compounds that showed a normal distribution for the total number of samples (i.e. PBDEs, HBB, dechloranes, PCDEs, PCTs and MeO-PBDEs), the temporal trends of their concentrations were also examined by linear regression analysis with the year as independent variable.

For means, standard deviations (SD) and box plots, concentrations below LOQ were given the LOD value and concentrations below LOD were considered to be 10% of the LOD. All statistical calculations were carried out using the statistical package IBM SPSS 20.

3. Results and discussion

Concentrations of FRs in muscle of striped dolphins are summarized in Table 2 (see Supporting Information for results of compound groups and individual compounds in each sample (Tables: S2, groups, S3, legacy FRs; S4, emerging HFRs; S5, OPFRs, and S6, MeO-PBDEs)). Due to the intrinsic variability of results from individuals that could differ in even a couple of decades of age, the standard deviation within a period is comparable to the contamination level. This variability of the data might obscure mild time trends. However, observing a difference

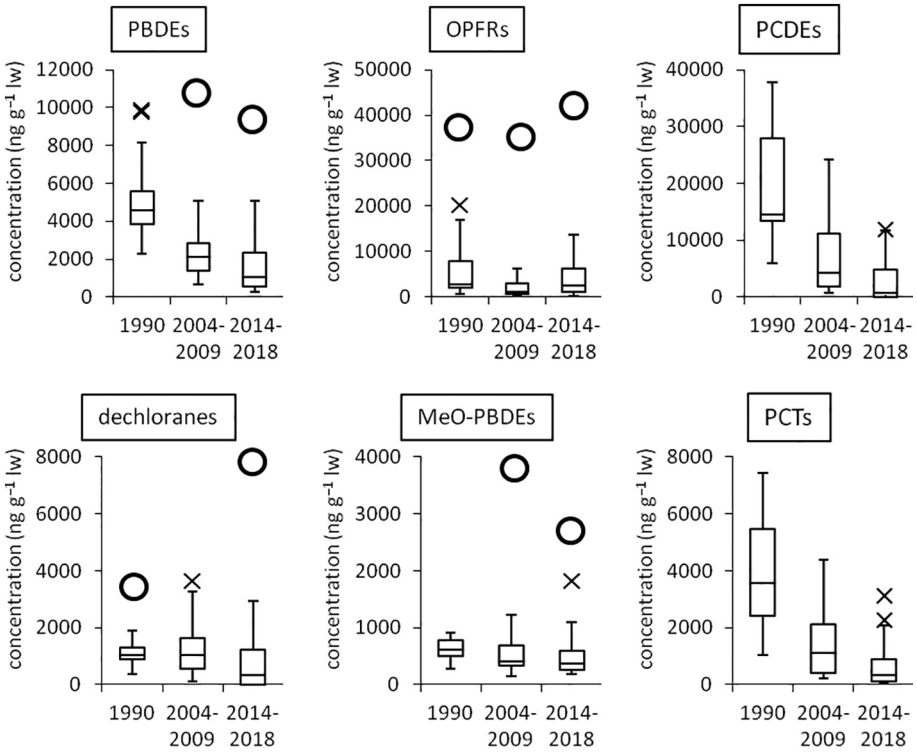

Fig. 1. Temporal trends of contaminants in dolphin muscle showed in the three periods. Two types of outliers are distinguished: mild outliers (X) and extreme outliers (O); the latter were excluded from the statistical analyses.

between periods despite this variability evidences that a change has clearly occurred.

3.1. Legacy BFRs

PBDE levels decreased from 5067 ± 2210 ng g^{-1} lw in 1990 to 2068 ± 2642 ng g^{-1} lw in 2004–2009 (Tukey test, $p < 0.001$) and no significant decrease has been observed from 2004–2009 to 2014–2018 (Table 2, Fig. 1). However, it must be taken into account that 1990 and 2004 are separated by 14 years, while 2009 and 2014 are only separated by 5 years. Therefore, assuming a regular decrease in contamination levels as reported in Canada (ECCC, 2016), a statistically significant change in concentrations would be easier to detect between 1990 and 2004–2009 as a bigger time gap would allow for greater differences in concentrations. Being the 2004–2009 and 2014–2018 periods as long as the separation between them, it made sense to analyse the trend along the whole three-decade period through a regression analyses. The result showed a highly decreasing of trend of PBDE concentrations of about 60% from 1990 to 2018. ($R = 708$, $p > 0.001$) (Fig. 1) and it is reasonable to think that the trend continues. The bans in Europe and North America in the early 2000s and the inclusion of PBDEs in the Stockholm Convention in 2011 may account for a decrease in the emission of these pollutants and, therefore, the amount detected in biota. These findings could validate the success of the prohibition.

But what do these levels represent in terms of risk to marine mammals? PBDEs have been related to disruption of thyroid functions, of neurobiological development and to foetal toxicity/teratogenicity in mammals (Alonso et al., 2014). Hall et al. (2003) established the threshold level for PBDEs in blubber (1500 ng g^{-1} lw) at which thyroid endocrine disruption in juvenile grey seals was observed. If we take this threshold as valid also for dolphins, we observe that 100% of analysed individuals from 1990 presented levels higher than 1500 ng g^{-1} lw, indicating high risk of suffering hyperthyroidism and associated thyrotoxicosis. The percentage drops to 73% of individuals from 2004 to 09. However, and despite the observed PBDE level decrease, and after 15 years of the ban of these pollutants in Europe, currently even 33% of dolphins are at risk of hyperthyroidism.

PBDE time trends keep a similarity with those of polychlorinated biphenyls (PCBs) in the same species and area. PCBs peaked between 1960 and the late 1970s before being prohibited and PCB levels in blubber of Mediterranean striped dolphin showed a marked decline from 1988, but then stabilized from 2001 to 2009, but with concentrations ranging from 7000 to 153,000 ng g^{-1} lw (Castrillon et al., 2010).

Regarding PBDE congeners, BDE-28, -47, -99, -100 and -154 were found in all the samples. BDE-153 and BDE-209 had high frequencies of detection (98 and 95%, respectively) and BDE-183 was found in 45% of the samples. BDE-47 was the main contributor to the PBDEs profile (≈40%) followed by BDE-154 (≈20%) and BDE-99 and -100 (≈14% each). BDE-154 is not a typical major contributor to the PBDE profiles. While no conclusive explanation could be found, it has been observed that several higher-brominated PBDEs (including half of the congeners in the Octa-BDE commercial mixture) debrominate preferably into BDE-154, while further debrominations do not clearly favour the other congeners (Zeng et al., 2010). On the other hand, since the concentrations of PBDE congeners did not follow a normal distribution (Shapiro Wilks $p < 0.05$), Spearman's bivariate correlation was calculated. All PBDEs were highly correlated with each other ($p < 0.01$) except the BDE-209, which was not correlated with BDE-28, -100, -154 and -183, but it was with the rest of congeners BDE-99, -153 (both $p < 0.05$) and − 47 ($p < 0.01$). It makes sense that BDE-209 be the less correlated congener as, differently from the other PBDEs, this congener is more commonly associated with sediments than biota.

In 28.6% samples, α-HBCD was quantifiable ranging from 1.99 to 4425 ng g^{-1} lw (550 ± 1233 ng g^{-1} lw). The number of samples with quantifiable concentrations in each period was 1, 7 and 4, in chronological order. Frequencies of detection in each period were 60.0, 73.3 and 83.3. Several tests were performed including either all samples or all samples except outliers or samples above LOD or samples above LOQ and no variation with time could be seen in any case ($p > 0.1$).

Dolphin blubber from the same and other species sampled from the south of Spain between 2004 and 2012 showed levels of HBCD in the same range as the dolphins from those years here studied and levels of PBDEs in dolphin blubber were in the lower half of our results (Barón et al., 2015a; Barón et al., 2015b). Those two studies included common dolphins (*Delphinus delphis*), bottlenose dolphins (*Tursiops truncatus*), pilot whales (*Globicephala melas*) and one of them also included Risso's dolphins (*Grampus griseus*) and striped dolphins.

3.2. Emerging HFRs

Just as α-HBCD and PBDEs showed different time trends, brominated emerging FRs can also be divided between unchanged and decreased levels. PBEB was detected in only 20% of the samples from 2004 to 2009 within the range of 0.86–3.20 ng g^{-1} lw, not far from its LOQ, 0.61 ng g^{-1} lw. With PBEB not being detected in 92.9% of the dolphins in the study, the only time trend observed was a constant absence of it. HBB was detected in a reduced and decreasing number of dolphins with time and in decreasing concentrations, 26.7% through 16.7% and 24.1 ± 41.4 ng g^{-1} lw through 1.46 ± 4.28 ng g^{-1} lw (Table 2). Taking into account only the values above the LOQ, results from the ANOVA showed that the concentrations of HBB decreased from 1990 to 2004–2009 ($p < 0.001$) and then remained steady until 2014–2018 ($p = 0.5$). However, when analysing the trend for the whole period (with year as the independent variable), a significant decrease was observed ($R = 0.942$, $p < 0.001$).

DBDPE showed the same pattern as HBB, albeit with higher frequencies of detection and concentrations: 100% through 75.0% and 85.6 ± 99.1 ng g^{-1} lw through 27.7 ± 74.0 ng g^{-1} lw (Table 2). With these higher values, the concentrations of DBDPE decreased from 1990 to 2004–2009 ($p < 0.01$) and then remained steady until 2014–2018 ($p = 0.15$).

Concentrations of dechloranes in Mediterranean dolphins also seem to have been steady for three decades ($p > 0.1$ for all Tukey post hoc comparisons between groups) at 1238 ± 1372 ng g^{-1} lw (Table 2, Fig. 1). The same regression analyses applied before to PBDEs was applied to dechloranes to provide further insight, showing no trends in the whole period ($R = 146$, $p = 0.37$) (Fig. 1). However, dechlorane levels appear to be lower in 2017–2018 than in the rest of years (Fig. 1). Whether this is the beginning of a decreasing trend or a one-time variation remains to be seen by samples to be collected during the next decade.

Dec 602 was detected in all the samples and accounted for 90.4 to 100% of the total dechlorane contamination (median = 100%). The range of concentrations of Dec 602 for all dolphins in this study is 55.2–7880 ng g^{-1} lw, while the range for the other dechloranes goes from not detected to 51.6 ng g^{-1} lw. Dec 602 was found in all the samples, Dec 603 and *anti*-DP were detected in 19.5% of the samples and Dec 604 and *syn*-DP in just 4.9% and 7.3% of them, respectively. Therefore, the time trend of dechloranes is namely a time trend for Dec 602. The median of the concentrations for each congener in the few quantifiable samples seem to somewhat decrease. Here they are expressed in ng g^{-1} lw and in chronological order. Dec 603: 30.1 to 2.1 to <LOD. Dec 604: 8.2 to <LOD to 0.6. *syn*-DP: 11.6 to 5.0 to <LOD. a*nti*-DP: 26.0 to 8.19 to 4.2. On the other hand, it is important to note that these compounds were detected in very few samples and at very low levels compared to Dec 602 in all periods.

Levels of dechloranes in dolphin blubber sampled from the south of Spain between 2004 and 2012 were an order of magnitude below those found in the present study (Barón et al., 2015a; Barón et al., 2015b). Levels of PCBs in biopsy samples have previously been reported to be lower than in stranded dolphins (Jepson et al., 2016). This could explain the difference between our results and the levels found in biopsy

samples. The smaller difference between North and South Mediterranean stranded striped dolphins might be related to the geographical habitat. As previously described, northern Spain is more industrialized than southern Spain and thus striped dolphins from the northwestern Mediterranean showed PCB concentrations that were double of those in their conspecific from the Alboran Sea (Marsili et al., 2018).

3.3. OPFRs

OPFRs appeared to have a steady concentration over time of 5811 ± 9831 ng g^{-1} lw (Table 2, Fig. 1). While in 1990 both OPFRs and PBDEs were the major contributors to the FR contamination in these dolphins, the decreasing levels of PBDEs have left OPFRs alone in that position. This makes sense as in 2006 OPFRs already doubled the amount of PBDEs used in Europe and in 2009 the Stockholm Convention started including PBDEs (Van der Veen and de Boer, 2012).

TBOEP was found in all samples from 1990 and 2004–2009 and in 66.7% from 2014 to 2018. TBOEP concentrations decreased from 1990 (2825 ± 3600 ng g^{-1} lw) to 2004–2009 (504 ± 416 ng g^{-1} lw) and 2014–2018 (132 ± 178 ng g^{-1} lw). TNBP and TCEP show steady levels at the orders of magnitude of 10^3 and 10^2 ng g^{-1} lw, respectively. EHDPP and IDPP were found in concentrations over 1000 ng g^{-1} lw in five and three samples, respectively. TDCPP and IPPP in less than 17% of the samples at concentrations below 20 ng g^{-1} lw, mostly below their LOQs. THP was found in one sample from 1990 at 112 ng g^{-1} lw.

Data about OPFRs in dolphins are scarce. There are currently two reports studying common dolphins that were found stranded in south of Spain between 2004 and 2010 (Sala et al., 2019) and three dolphin species from the southwestern Indian Ocean (*Delphinus capensis*, *Sousa plumbea* and *Tursiops aduncus*) accidentally caught in shark nets between 2012 and 2015 (Aznar-Alemany et al., 2019). Like in the present study, the samples were muscle tissue of dead individuals. Levels in the common dolphins ranged from 69.5 to 2939 ng g^{-1} lw, with a mean value of 994 ng g^{-1} lw. Levels in the dolphins from the Indian Ocean ranged from 1630 to 31,861 ng g^{-1} lw, with a mean value of 10,452 ng g^{-1} lw. The levels reported in this article for striped dolphin were slightly higher than the levels in common dolphin, sampled 800 km south from our location. The difference in species and location might account for this. The range of levels in the Indian Ocean reached a higher maximum concentration. Levels of PBDEs in those dolphins were also higher than one would expect and further research is required to assess the reason for that difference. Additionally, and coinciding with the present work, TBOEP presented the highest frequencies of detection in both studies, 77 and 82%, respectively.

3.4. MeO-PBDEs

Finally, one could expect MeO-PBDEs to show stable levels through time as they are natural compounds independent from anthropogenic pressure and legislation. In fact, the ANOVA did not show variation in the three periods ($p > 0.1$, for all Games-Howell post hoc comparisons between groups). Agreeing with previous studies with delphinids from the Mediterranean Sea (Barón et al., 2015a; Barón et al., 2015b), Brazil (Alonso et al., 2012) and South Africa (Aznar-Alemany et al., 2019), the most frequently occurring congeners were 6-MBDE-47 (124–2839 ng g^{-1} lw, in 100% of the samples) and 2-MBDE-68 (23.4–894 ng g^{-1} lw, 95.2%). Additionally, 5-MBDE-100 and 4-MBDE-100 were found in 83.3 and 59.5% of the samples at concentration one or two orders of magnitude lower. Total concentrations of MeO-PBDEs reported in blubber of the Mediterranean delphinids sampled between 2004 and 2012 were in the same range.

3.5. Non-targeted compounds: PCTs and PCDEs

A high-resolution mass spectrum may be visualized by constructing a Kendrick mass defect plot. Visual interrogation of such a plot enables efficient discovery and annotation of (unknown) chemical compounds in complex environmental samples. Kendrick realised that by replacing the International Union of Pure and Applied Chemistry (IUPAC) mass scale (C = 12.000 Da) with the Kendrick scale (CH_2 = 14.000 Da), organic ions belonging to homologous series could readily be identified on the basis of their mass defect (Kendrick, 1963). The conversion is achieved applying a 14/14.01565 factor to IUPAC mass and the utility of this approach is demonstrated by the fact that Kendrick mass defect of methyl, ethyl and propyl naphthalene is 0.9195 Da. The same approach also works well for Cl, Br congeners (Taguchi et al., 2010; Jobst et al., 2013) and CF_2 homologues (Myers et al., 2014) using other non-standard mass scales. The Kendrick mass defect plot represents the Kendrick mass defect of each mass spectral peak (y axis) plotted against the nominal mass (x axis) (Hughey et al., 2001). In this study, the H/Cl mass scale proposed by Taguchi et al. (2010) was employed. Consequently, Br/Cl congener series appear as a band of peaks parallel to the x axis in a specific region of the mass defect plot. Additionally, isotope patterns, like those of bromine and chlorine, are easily recognizable.

Due to the complexity of the GC × GC-HRQTOFMS analysis, five samples of each period were selected for the analysis of non-targeted compounds. Two groups of peaks showing chlorine isotope patterns and separated 34 mass units from each other were present in the mass defect plots of all of the samples (Fig. 2, Table S7).

With $C_{18}H_{14-x}Cl_x$ (x = 5–7) as a formula, compound group A was thought to correspond to polychlorinated terphenyls (PCTs). PCTs are similar to PCBs, in terms of chemical properties and toxicity (Jensen and Jørgensen, 1983). Long-term toxic effects of PCTs in animals have shown to be of most importance. PCTs may cause liver damage, including tumours, and disturb the hormonal, immunological and reproductive systems. Few publications address the occurrence of PCTs in the Mediterranean Sea. They were detected in shellfish collected between 1989 and 1991 from the Catalan coast in levels lower than PCBs' (Galcerán et al., 1993a; Galcerán et al., 1993b). PCTs were also found in clams, mussels, tuna and salmon purchased in markets in Madrid (Spain) (Fernández et al., 1998).

Compound group B was identified as having the formula $C_{12}H_{10-x}OCl_x$ (x = 4–8), which likely corresponded to polychlorinated diphenyl ethers (PCDEs). Due to their higher log K_{ow} values, PCDEs bioaccumulate in organisms at a much higher level than hydroxyl PCBs (Koistinen, 1998).

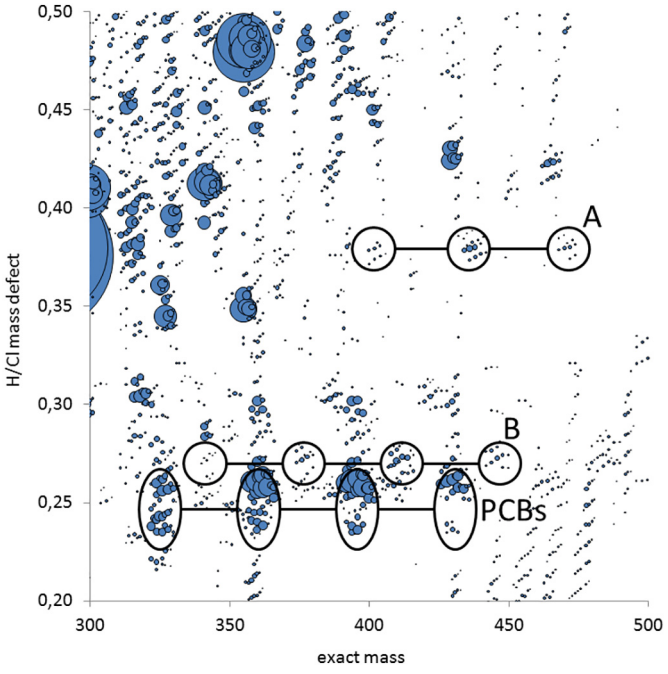

Fig. 2. H/Cl mass defect plot of a sample: A and B are unknown compounds.

Despite their connection with PCBs and PBDEs, the occurrence of PCDEs is not as well documented as that of the other compounds. PCDEs might also be environmentally significant due to their conversion into polychlorinated dibenzodioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) in photolytic reactions and industrial processes (Becker et al., 1991; Domingo, 2006). They do not seem to have been documented in the Mediterranean Sea yet.

Two PCT mixtures (di- to penta-PCTs Aroclor 5432, hepta- to undeca-PCTs Aroclor 5460) and PCDEs with different levels of chlorination (tetra-CDE-77, penta-CDE-99, hexaCDE-153) were purchased to confirm the identity of the unknown compounds. Full scan at low and high energy were registered for all standards and samples. Runs at low and high energy provided the molecular ion of the analytes and a confirmation fragment (generally by loss of two chlorine atoms), respectively. The suspected compounds matched the retention times and the mass spectra of the standards (Fig. S1). Lacking the corresponding standards, hexa-CTs, hepta-CDEs and octa-CDEs were identified by their molecular mass, matching their mass spectra with modelled mass spectra and at times of retention consistent with the regular separation observed between the available congeners.

Instrumental blanks showed no compounds. Hepta- and octa-CDE and tri-, tetra-, penta-, hexa- and deca-CT showed in one, two or three procedure blanks at 0.59% ± 0.96 of the samples' response.

After the confirmation of PCTs and PCDEs, semi-quantitative determination was performed (Table 2). An external calibration line using the labelled internal standard with the closest retention time was used. As PCDE standards of different chlorination degree were purchased separately, it was assumed that all PCDEs of the same chlorination degree would have the same analytical response and equal to the purchased standard. Lacking hepta- and octa-CDE standards, having observed that sensitivity seemed to increase with the degree of chlorination and bearing in mind that this was a semi-quantification with the aim to set a tentative order of magnitude and detect temporal trends, hepta- and octa-CDE were semi-quantified using the hexa-CDE calibration. Regarding PCTs, which came in two Aroclor mixtures, it was assumed that all degrees of chlorination in a mixture had the same analytical response; therefore, the proportion of each group's area relative to the sum of areas equalled the proportion in the composition of the mixture. It was observed that the semi-quantification of hexa-CT, which was not included in any of the mixtures, was similar using penta- and hepta-CT (average difference of 2.7%). Therefore, it was semi-quantified as an average of both results bearing in mind the tentative order of magnitude and the focus on temporal trends. Values were rounded as they are an indication of magnitude rather than an exact quantity. The determined concentrations are probably somewhat lower than the true values due to incomplete recovery of the compounds in the extraction. See Supporting Information for results of individual compounds in each sample (Tables: S8, PCTs, and S9, PCDEs).

Tri-CTs were found in 23.8% of the samples, deca- and nona-CTs were in 66.7 and 71.4% of them, tetra- to octa-CTs were in over 95% of the samples. Hexa-CTs were semi-quantified as the mean of the values obtained using the calibration lines of penta- and hepta-CTs (most values differed by less than 30%).

Tetra-CDEs were not detected, penta-CDEs were found in three samples and hexa-CDEs were in all but the sample from 2018. Hepta-CDEs were present in 38.1% of the samples and octa-CDEs were in all but the sample from 2018. The total peak group of penta- and hexa-CDEs was semi-quantified with their respective standard assuming a similar response for the congeners with the same degree of chlorination. Hepta- and octa-CDEs were semi-quantified using the calibration line of hexa-CDEs.

Both groups of compounds (PCTs and PCDEs) showed clearly declining levels between 1990 and 2014–2018 ($p < 0.01$, Games-Howell post hoc comparison, Fig. 1). Moreover, both groups showed a significant decrease through all periods (linear regression applied to concentrations versus year: $R = 56$, $p < 0.001$).

In 1990, PCT levels were comparable to PBDE levels. In 2014–2018, they are comparable to dechloranes and MeO-PBDEs. In 1990, PCDE levels were roughly three or four times the levels of OPFRs and PBDEs. In 2014–2018, they seemed to be somewhere between the two families of FRs.

Studies on PCTs in marine biota are very scarce. They have been reported in Spanish seafood samples ranging between 9.84 and 17.6 ng g^{-1} wet weight (ww) in mussels, 1.24–24.2 ng g^{-1} ww in clams, 21.1–64.2 ng g^{-1} ww in salmon and 63.4–259 ng g^{-1} ww in tuna. Expressed in ww, the present semi-quantitative results in Mediterranean striped dolphins sampled in 1990 ranged from 6 to 36 ng g^{-1} ww. These levels were in the same order of magnitude as those of the other Mediterranean organisms. PCTs were reported in 1981 in grey seals from the Baltic Sea at 500–1000 ng g^{-1} ww (Renberg et al., 1978). Levels in fat of gulls from the Mediterranean Sea (1978) and the Baltic Sea (1980) were 610–10,510 ng g^{-1} ww (range) and 1800 ng g^{-1} ww (mean), respectively (Vannucchi et al., 1978; Falandysz, 1980). If the similar concentrations in gulls were an indication that the order of magnitude of the environmental contamination in both seas was comparable, similar concentration may be found in marine mammals. Assuming a broad comparison of grey seals with striped dolphins as marine mammals and taking into account the decline in levels observed in the present study, PCT semi-quantitative concentrations in the dolphins from 1990 are consistent with the concentrations in the grey seals from 1987.

Studies on PCDEs in marine biota are just as scarce. Total concentrations of PCDEs in seals from Lake Saimaa (Finland, 1991–1992) and Baltic seals (1993) were 220–460 ng g^{-1} lw and 30–380 ng g^{-1} lw (Koistinen et al., 1995; Koistinen et al., 1997). Those levels were much lower than the levels obtained for the Mediterranean dolphins from 1990, 6000–38,000 ng g^{-1} lw. Other compounds have previously been found at high levels in the Mediterranean Sea. A study on the impact of PCBs on cetaceans in European waters reported that striped dolphins and other species around the Iberian Peninsula had their mean and median PCB concentrations among the highest published for cetaceans globally (Jepson et al., 2016). PBDEs in seafood showed higher levels in the Mediterranean Sea than in other European waters and high levels of pyrethroid pesticides have been reported in striped dolphins from the south of the Iberian Peninsula (Aznar-Alemany et al., 2017a; Aznar-Alemany et al., 2017b).

A PCB toxicity threshold for the onset of physiological impacts in dolphins is 10 mg kg^{-1} lw (Hall et al., 2006), and a higher threshold was reported for reproductive impairment in ringed seals (*Phoca hispida*) from the Baltic Sea at 41 mg kg^{-1} lw (Helle et al., 1976). If PCTs are similar to PCBs in terms of toxicity (Jensen and Jørgensen, 1983), their levels were always below the lower threshold. However, assuming some similarity with PBDEs, approximately 82% of dolphins exceeded the PBDE threshold (1500 ng g^{-1} lw) in 1990 compared with 17% in 2014–2018. Regarding PCDEs, over 90% of the individuals exceeded the lower PCB and the PBDE thresholds in 1990, whereas in 2014–2018 the percentage decreased to 17% and 42%, respectively.

4. Conclusions

In this study, target and non-target analysis were carried out, obtaining contamination levels from a wide variety of halogenated and organophosphate contaminants in dolphin samples from the Mediterranean Sea. For some compounds such as PBDEs, previous studies have been already published. However, for other families of emerging compounds such as OPFRs, or less studied pollutants such as PCTs and PCDEs, our results are the first ones reported in this type of samples. Furthermore, we have been able to characterize time trends of the different compounds.

PBDEs and OPFRs showed the highest concentrations in the orders of magnitude of 10^3–10^5 ng g^{-1} lw. However, while PBDE levels have decreased about 60% from 1990 to 2018, probably due do the relevant

legislation, OPFRs showed steady concentrations over time becoming the predominant family of these FRs to take into account for environmental management. Dechloranes contamination was mainly attributed to Dec 602. Dec 602 reached maximum concentrations in the low 10^3 ng g^{-1} lw values. Although Dec 602 levels seemed stable, lower levels in 2017 and 2018 hinted at a possible decrease that should be assessed in future studies. PBDEs, OPFRs and Dec 602 were found in all samples, as well as natural MeO-PBDEs. Being naturally occurring, hence independent from human activity, MeO-PBDEs showed steady levels below 1000 ng g^{-1} lw. Legacy α-HBCD and emerging BFRs were detected in fewer samples and at lower levels. No time trend was observed for α-HBCD or PBEB, the latter mostly undetected. On the other hand, concentrations of DBDPE and HBB decreased between 1990 and 2018. Finally, PCTs and PCDEs showed clearly declining levels between time period evaluated.

Although there is a trend of decreasing concentration levels of certain pollutants over time, they still remain a health risk for dolphins. For instance, and currently, 33% of dolphins are at risk of hyperthyroidism due to PBDE levels. Similarly, from 17 to 42% of dolphins still have PCDE risk levels. The present study suggests that we will have to wait at least another decade for PBDEs and PCDEs do not pose a threat to Mediterranean striped dolphins.

CRediT authorship contribution statement

Òscar Aznar-Alemany: Conceptualization, Formal analysis, Investigation, Writing – Original Draft, Writing – Review & Editing, Visualization. **Berta Sala:** Methodology, Formal analysis (OPFRs). **Karl J. Jobst:** Methodology, Formal analysis (Non-targeted analysis), Investigation, Writing – Original Draft. **Eric J. Reiner:** Methodology, Validation, Investigation, Resources, Supervision, Project administration, Funding acquisition. **Asunción Borrell:** Conceptualization, Resources, Writing – Original Draft, Writing – Review & Editing, Supervision, Project administration, Funding acquisition. **Alex Aguilar:** Resources, Writing – Original Draft, Writing – Review & Editing, Supervision, Project administration, Funding acquisition. **Ethel Eljarrat:** Conceptualization, Methodology, Validation, Investigation, Resources, Writing – Original Draft, Writing – Review & Editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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